Life Sciences 118 (2014) 219–225

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Endothelin-1 overexpression and endothelial nitric oxide synthase knock-out induce different pathological responses in the heart of male and female mice

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ARTICLE INFO

Article history:
Received 24 October 2013
Accepted 4 December 2013
Available online 17 December 2013

Keywords:
Endothelin-1
Nitric oxide
Cardiac function
Sexual dimorphism

ABSTRACT

Aims: The nitric oxide and endothelin systems are key components of a local paracrine hormone network in the heart. We previously reported that diastolic dysfunction observed in mice lacking the endothelial nitric oxide synthase (eNOS−/−) can be prevented by a genetic overexpression of ET-1. Sexual dimorphisms have been reported in both ET-1 and NO systems. Particularly, eNOS−/− mice present sex related phenotypic differences.
Main methods: We used the ET-1 transgenic (ET+/+), eNOS−/−, and crossbred ET+/+eNOS−/− mice, and wild type controls. We measured cardiac function by heart catheterization. Cardiac ventricles were collected for histological and molecular profiling.
Key findings: We report here that (i) the level of ET-1 expression in eNOS −/− mice was elevated in males but not in females. (ii) Left ventricular end-diastolic blood pressure was higher in male eNOS −/− mice than in females. (ii) eNOS −/− males but not females developed cardiomyocyte hypertrophy. (iv) Perivascular fibrosis of intracardiac arteries developed in female ET+/+ and eNOS −/− mice but not in males. Additionally, (v) the cardiac expression of metalloprotease-9 was higher in eNOS−/− males compared to females. Finally, (vi) cardiac proteome analysis revealed that the protein abundance of the oxidative stress related enzyme superoxide dismutase presented with sexual dimorphism in eNOS−/− and ET+/+ mice.
Significance: These results indicate that the cardiac phenotypes of ET-1 transgenic mice and eNOS knockout mice are sex specific. Since both systems are key players in the pathogenesis of cardiovascular diseases, our findings might be important in the context of gender differences in patients with such diseases.

Introduction

Endothelin-1 (ET-1) and nitric oxide (NO) are crucial components for cardiac function and morphology. Both systems belong to a tightly regulated hormonal network (Slowinski et al., 2007) and a deregulation of the balance between ET-1 and NO occurs in many cardiovascular diseases (Rossi et al., 2001). Mice lacking the main source of NO, the endothelial NO synthase (eNOS) (eNOS−/−) develop high blood pressure and age related cardiac hypertrophy (Li et al., 2004). ET-1 transgenic overexpression in mice leads to an elevation in expression of some extracellular matrix (ECM) proteins in heart but is not sufficient to alter dramatically cardiac phenotype and to elevate blood pressure due to probable compensatory effects of the NO system (Hocher et al., 2004). We recently generated crossbred animals of ET+/+ and eNOS−/− mice to disrupt these compensatory effects in ET+/+ mice. In contrast to this hypothesis, the overexpression of ET-1 could prevent the cardiac diastolic dysfunction observed in eNOS−/− mice (Vignon-Zellweger et al., 2011).

Sexual dimorphism has been observed in both NO and ET systems in experimental models as well as in humans. eNOS activity is enhanced by estrogen at the transcriptional and posttranslational level (Kauser and Rubanyi, 1997). When male eNOS−/− mice develop hypertrophy, the females do not and a premature death is observed only in eNOS−/− males (Li et al., 2004). In rats, eNOS protein levels are significantly higher in the renal medulla of females compared with males (Neugarten et al., 1997). A higher level of circulating ET-1 has been measured in men than in women and analysis in transsexual individuals indicated that plasma ET-1 levels might be regulated by sexual hormones (Polderman et al., 1993). The ratio and the density of ET receptors are different in men and women leading to a different regulation of the contractile response (Ergul et al., 1998). Pharmacological studies performed in humans targeting the ET-B receptors have shown that the contribution of this receptor to vascular tone might differ between women and men (Kellogg et al., 2001). One reason for the lack of hypertension in

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http://dx.doi.org/10.1016/j.lfs.2013.12.003

female ETB deficient rats might be a higher eNOS activity compared to males (Taylor et al., 2003). This higher activity could be a response to a positive control of eNOS expression by sex hormones like 17-beta-estradiol (Sumi and Ignarro, 2003). Finally, castration in male ET+/+ mice ameliorates renal functions, indicating that androgens control ET-1 actions (Kalk et al., 2009).

In this article, we explored the differences in cardiac physiology and morphology observed between male and female ET+/+, eNOS−/− and crossbred ET+/+eNOS−/− mice.

Material and methods

Mice

We used the previously described ET+/+, eNOS−/−, ET+/+ eNOS−/− mice and the wild type (WT) controls (Quaschning et al., 2007). Female eNOS−/− mice and male homozygous ET-1 transgenic mice were originally used to generate the crossbred animals (Quaschning et al., 2007). Several backcrossing has been performed to ensure a homogenous genetic background between the four strains. Genotypes were confirmed by PCR on genomic DNA. The following primers specific for the human endothelin-1 transgene were used to ensure the presence or absence of the transgene (forward: CCCCATTCTA AGCATAGGGGC; reverse: AGCCAGTGAAGATGGTTGGGG). Primers specific for the neomycin resistance (NeoR) cassette inserted in the eNOS gene (forward: TTGTCAAGACCGACCTGTCC; reverse: ACAAGACCGGCT TCCATCCG) and primers specific for the wild type eNOS gene (forward: AGGACATATGTTTGTCTGCGG and reverse: CTGAGGACTGCACCTGTTCA) were used to verify the knock-out of the eNOS gene. Typical images of the amplification loaded on gel can be seen in Supplementary Fig. 1. Mice were kept under standard conditions, fed on a standard rodent diet and received water ad libitum. Mice were sacrificed at the age of nine months. Hearts were harvested, the atrium was removed and the ventricles were washed and fixed in paraformaldehyde or snap frozen in liquid nitrogen.

ET-1 gene expression

RNA was extracted from the cardiac ventricles and qRT-PCR was performed as previously described (Vignon-Zellweger et al., 2011). The primers were designed to amplify both the mouse endogene and human transgene.

Blood pressure

Systolic blood pressure was measured by the tail cuff method in awaken and trained mice. Left ventricular systolic and end-diastolic pressure (LVEDP) was measured by heart catheterization as previously described (Vignon-Zellweger et al., 2011).

Histology

Cardiac ventricles were prepared, washed in saline, fixed in 4% buffered paraformaldehyde, and dehydrated in a graded alcohol series. Following xylene treatment, organs were embedded in paraffin blocks and cut in serial sections (5 μm) using a Microm HM230 Microtom. Slices were dewaxed with xylol and descending ethanol series and stained with 0.1% Sirius red in saturated aqueous picric acid. Pictures from the left ventricle and septum were taken with 200× magnificence. For interstitial fibrosis measurement, Sirius red-positive area of every picture was quantified using the image processing software ImageJ (shareware from the NIH, USA) with a threshold derived from a subset of randomly chosen pictures. The Sirius red-positive area was expressed as a percentage of total tissue area. For perivascular fibrosis measurement, two independent investigators, who were blinded to the study groups, graded the fibrosis around cardiac arteries using 5 different grades based on the relation between media-diameter and the diameter of the surrounding collagen ring of the adventitia (grade 1 = diameter of collagen-ring-wall < 25% in comparison to diameter of media-wall; grade 2 = 25–50%; grade 3 = 50–75%; grade 4 = 75–100%; grade 5 = >100%). Cardiac perivascular fibrosis was then expressed as the mean value of the obtained grades.

Immunohistochemistry

For immunohistochemical staining, 6 μm thick frozen sections of the cardiac ventricles from nine months old animals were used. For detection of matrix proteins, rabbit anti-MMP2 (Chemicon AB19167), anti-MMP9 (Chemicon AB19016), anti-Col I (Chemicon AB765P) and anti-Col III (Calbiochem 234189) primary antibodies were diluted 1/200 and anti-TIMP4 (Chemicon AB19168) 1/25 in an avidin/biotin normal serum blocking solution and incubated on slices for 1 h at room temperature. After wash steps, binding of the antibodies was revealed using the ABC Kit elite Vectastain Rabbit Ig (Vector PK6101). The same procedure omitting the primary antibody was used as negative control. Slides were photographed with a digital camera plugged to the microscope with 200× magnificence. The relative intensity of the signal was measured on 8 bit pictures using the threshold function of the software ImageJ (version 1.37V).

Proteomics

Cardiac proteins were extracted and separated by two-dimensional electrophoresis and the gels were analyzed as described previously (Schwab et al., 2011). Briefly, whole hearts (n = 6–9) were homogenized and total proteins were extracted. The proteins were first separated according to their isoelectric point and second according to their molecular weight on a polyacrylamide gel. The proteins separated on the gels were silver stained. Each spots correspond to a protein species with a specific isoelectric point and molecular weight. The intensity of the spots was analyzed using specific software. The spots of interest were excised from the gel and digested. The peptide mixtures were applied to an electron spray ionizer coupled to a mass spectrometer for sequencing. This step was performed by the Proteome Factory (Proteome Factory AG, Berlin, Germany) according to their protocols. The sequences of the peptides were matched to the database SwissProt to identify the proteins.

Statistical analysis

All data are expressed as means ± SEM. Statistical analysis was performed using the Mann–Whitney U-test or the Student's T-test as mentioned in the figure captions. A value of $p < 0.05$ was considered to be statistically significant.

Results

Blood pressure and cardiac function

Systolic blood pressure measured by the tail cuff method was higher in both eNOS−/− and ET+/+eNOS −/− mice independently of sex. Left ventricular systolic blood pressure measured by heart catheterization showed no differences, apart from a tendency to higher pressure in male eNOS−/− compared to male ET+/+eNOS−/− mice (Fig. 1). This difference was absent in females. The left ventricular end-diastolic pressure was however elevated in eNOS−/− mice both males and females but the elevation was two-times stronger in males compared to females (increase in males: 205%, increase in females: 95%) (Fig. 1). LVEDP was thus significantly higher in eNOS −/− males compared to males from other genotypes and to eNOS −/− females.

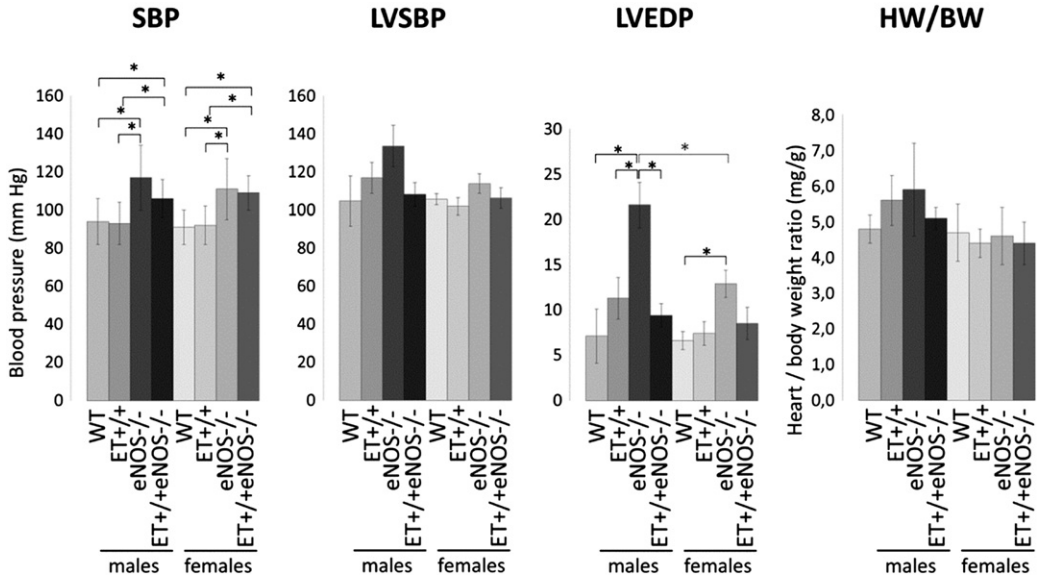

Fig. 1. Systolic blood pressure measured by the tail cuff method (SBP, n = 12–18), left ventricular systolic blood pressure (LVSBP) and end-diastolic pressure (LVEDP) (n = 6) measured by heart catheterization (mm Hg) and heart weight to body weight ratio (mg/g) (n = 12–18). Mean ± sem. Student's T-test *p < 0.05.

Cardiac morphology

Heart weight to body weight ratio measurement did not reveal any significant differences neither between the genotypes nor between males and females (Fig. 1). However, cardiomyocyte diameter measurement indicated a cardiac hypertrophy in the eNOS−/− male mice compared to WT and ET+/+ male mice, which was absent in the females (Fig. 2). Moreover, whereas no sex-related differences were seen in WT mice, the cardiomyocyte diameter was larger in ET+/+ and eNOS−/− males compared to females. These differences were no longer observed in crossbred ET+/+eNOS−/− mice (Fig. 2).

Cardiac fibrosis

ET+/+eNOS−/− male and female mice developed interstitial fibrosis compared to WT mice (Fig. 3A). No difference was observed between ET+/+, eNOS−/− and WT mice. All genetically modified females developed perivascular fibrosis whereas only ET+/+eNOS−/− males did

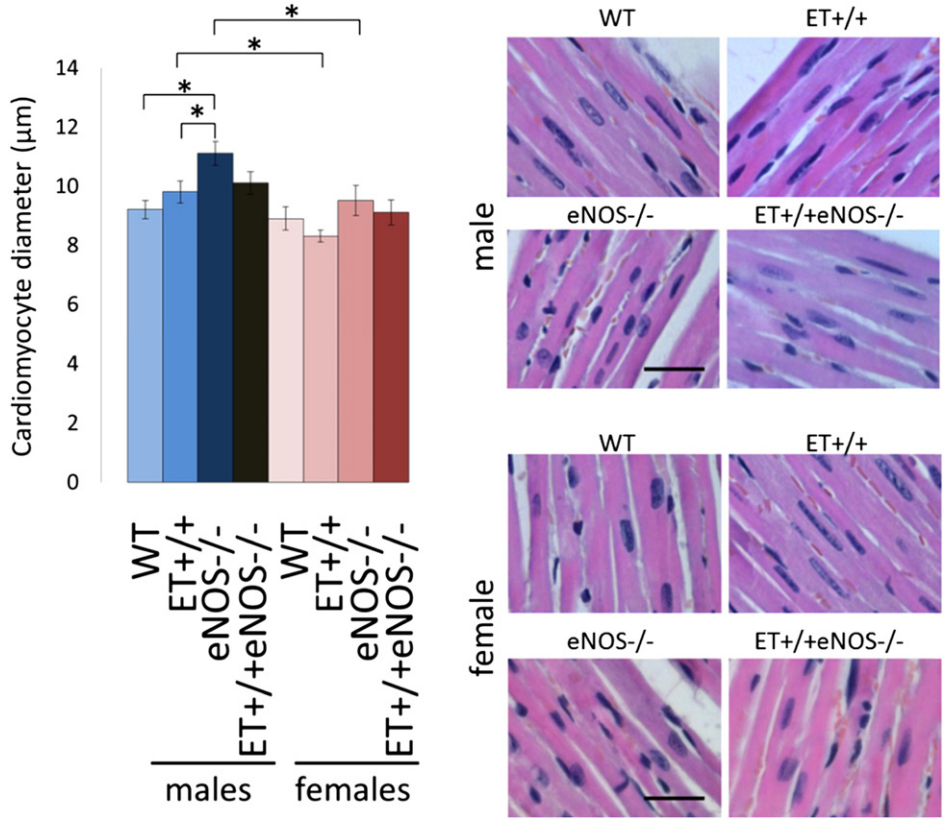

Fig. 2. Cardiomyocyte diameter (µm) measured on hematoxylin–eosin stained tissue slices. Mann Whitney U-test *p < 0.05. Representative images are presented; bar represents 10 µm.

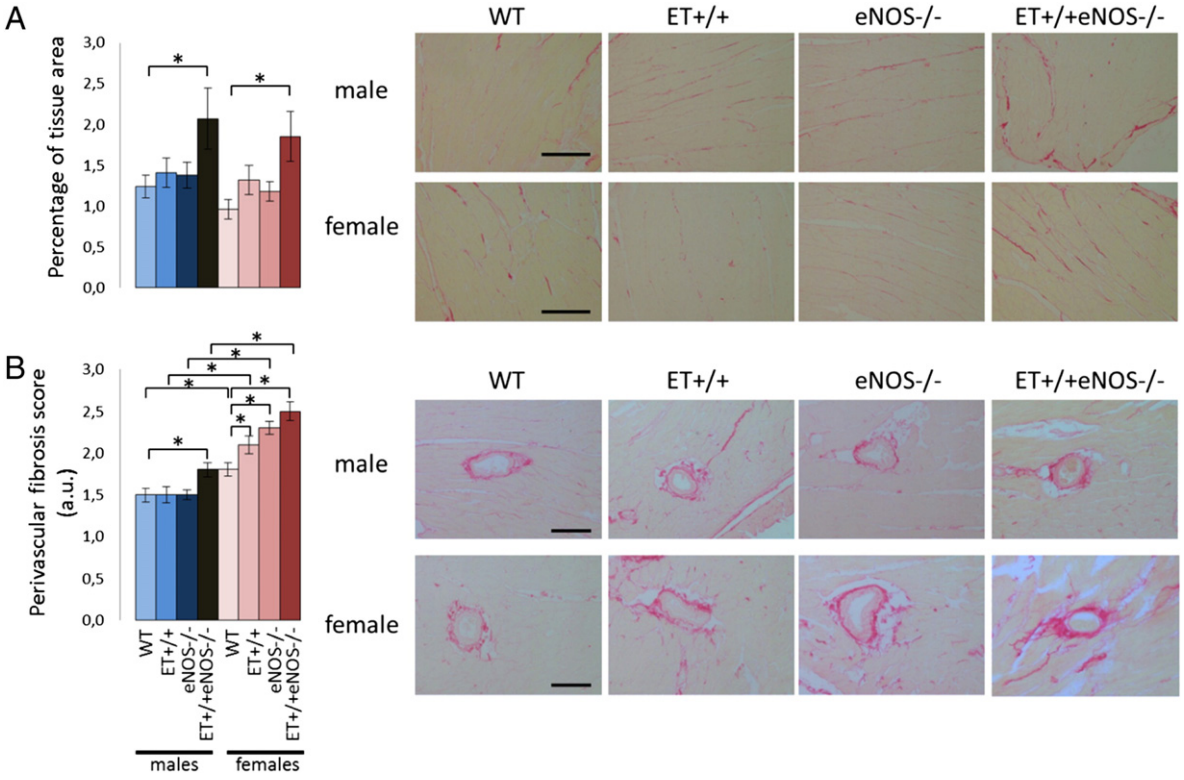

Fig. 3. Cardiac interstitial (A) and perivascular (B) fibrosis score measured on Sirius red stained slices (n = 9–11). Mann Whitney U-test *p < 0.05. Representative images are presented; bar represents 100 μm.

(Fig. 3B). Perivascular fibrosis score was more important in females compared to males in every genotype (Fig. 3B).

Gene expression and protein abundance

The level of messenger RNA of ET-1 in cardiac ventricles was measured by real-time PCR. It was doubled in mice overexpressing ET-1, both males and females. In eNOS−/− and ET+/+eNOS−/− male mice, the ET-1 mRNA level was also two-times higher than in WT mice but this was not the case in female mice (Fig. 4).

The expression of key proteins involved in the fibrotic process was investigated by immunohistochemistry. Female WT expressed less collagen I than male WT, and this relation was inverse in ET+/+eNOS−/− mice (Fig. 5A; B). Collagen III was modestly up regulated in ET+/+eNOS−/− mice compared to WT, both sexes taken together (Fig. 5). MMP2 expression and MMP9 expression were similar between the genotypes. Both eNOS−/− and ET+/+eNOS−/− females expressed less MMP9 than the males (Fig. 5A; C). No significant differences were observed in TIMP-4 expression between the groups (data not shown).

The detailed analysis of the cardiac proteome has been presented previously (Vignon-Zellweger et al., 2011). Here we show that the number of protein spots for which the abundance changed compared with the WT mice was different in males and females (Fig. 6A). This indicated that the proteome of the mice was differently regulated in males and females because of the genetic manipulations. In particular, the abundance of the antioxidant enzyme SOD1 was higher in females compared with males (Fig. 6B).

Discussion

In the present study, we have compared the cardiac phenotype of male and female WT, ET+/+, eNOS−/−, and ET+/+eNOS−/− mice. We have showed that male and female mice are not equal in regard to the consequences of the genetic knock-out of eNOS. The severity of the diastolic dysfunction was twice less in females than in males. Furthermore, the sexual dimorphism observed in this study was underlined by the absence of cardiac hypertrophy in eNOS−/− females.

The development of LV dysfunction and myocardial hypertrophy is more rapid in spontaneously hypertensive male rats than in females (Tamura et al., 1999). In rats with ascending aorta constriction, heart failure characterized by diastolic dysfunction develops faster in males than in females (Douglas et al., 1998). Male but not female AT-1

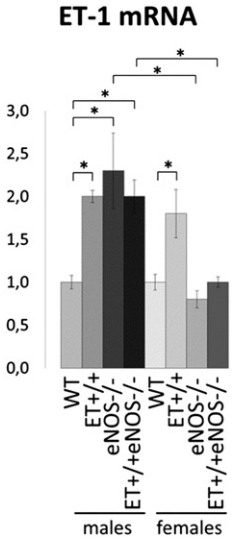

Fig. 4. ET-1 mRNA expression normalized to HPRT expression measured by real-time PCR (n = 5–6). Mean ± sem. Student's T-test *p < 0.05.

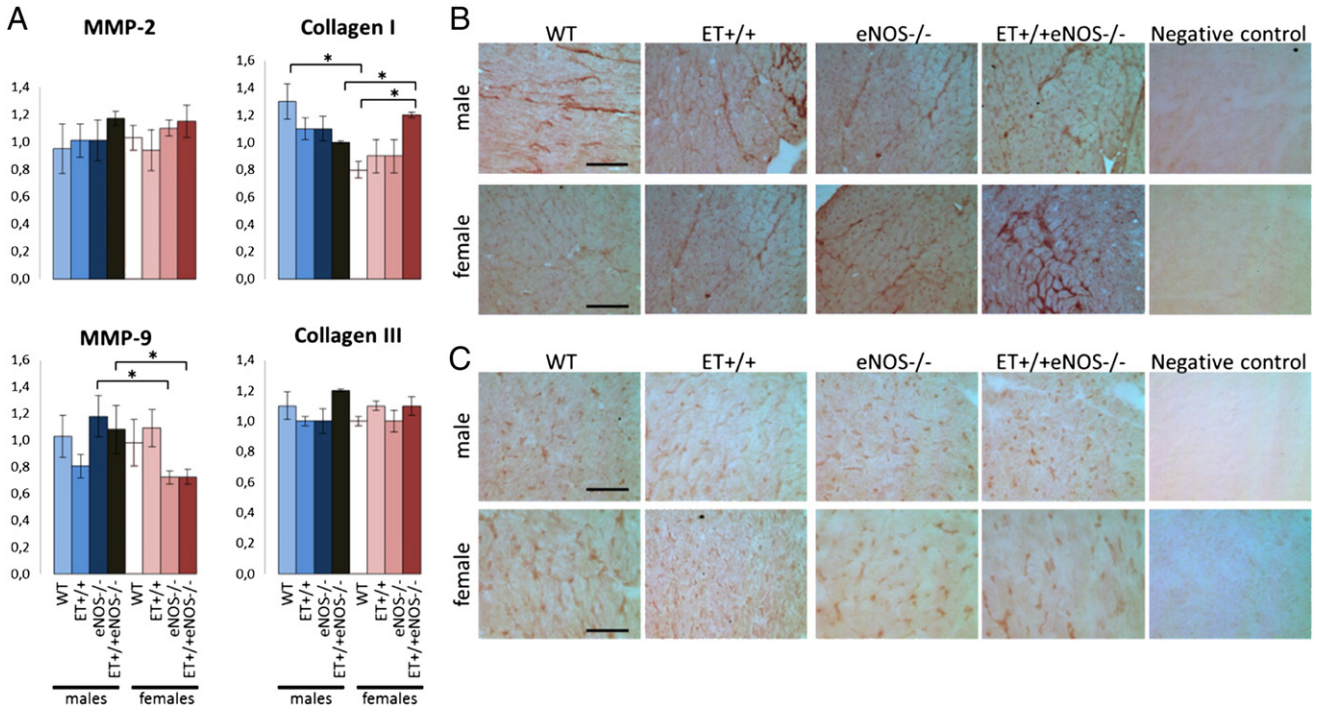

Fig. 5. A. Metalloprotease 9 (MMP9), metalloprotease 2 (MMP2), collagen I and collagen III expression in the cardiac left ventricle measured as percentage of positive immunostaining signal of tissue area. (n = 6–9). Mean ± sem. Mann Whitney U-test *p < 0.05. Representative images are showed for Collagen I (B) and MMP9 (C); negative controls without primary antibody are showed. Bar represents 100 μm.

receptor deficient mice develop cardiac hypertrophy in a model of myocardial infarction (Bridgman et al., 2013).

Studies in rats and mice have shown that the lack of estrogens has a deleterious effect on cardiac diastolic function (Delbeck et al., 2011; Groban et al., 2008; Wang et al., 2013). In line with this, elderly women are more susceptible to develop diastolic dysfunction than men (Ahtarovski et al., 2013; Graça et al., 2014), which might indicate that estrogens play a role in the development of this pathology. Our female mice could adapt better from the absence of eNOS than the males. The underlying mechanisms of this phenomenon are so far unknown. We hypothesized that in female mice NO might be generated by non-eNOS source of NO. The cardiac protein abundance of iNOS was however not different between WT and eNOS−/− mice (in both sexes) (Vignon-Zellweger et al., 2011). This corroborates previous observations in the lung of eNOS−/− mice, where iNOS levels are similar compared to WT mice despite a protection observed in female mice in terms of vascular remodeling (Miller et al., 2005). The better outcome of the eNOS−/− females might rely on eNOS-independent protective effects of estrogens.

Sex hormones control the expression of L-type Ca^{2+} channels, which are central actors of the depolarizing current in myocytes and therefore crucial for heart function (Valverde et al., 2011; Yang et al., 2012). The potential differential expression of these channels in our mice might explain our observations. Sex hormones have been shown to control enzymes implicated in sugar and fatty acid metabolism (Huss et al., 2004). We have seen that the abundance of key enzymes of the glycolytic pathway may be differently regulated in male and female eNOS −/− mice (Vignon-Zellweger et al., 2011). Female eNOS−/− mice might favor the utilization of glucose opportunely to prevent reduction of heart function (Fragasso et al., 2007).

Importantly, the better diastolic function in female eNOS−/− mice compared to males, might be explained by the lower expression of ET-1 measured in females. Blocking the endothelin pathway has been shown beneficial for heart function in several animal models (Sakai et al., 1996; Yamauchi-Kohno et al., 1999). It is however seemingly contradictory that these arguments tending to prove the negative effects of ET-1 on cardiac function are refuted in our own study by the fact that overexpression of ET-1 prevented the development of cardiac dysfunction in the eNOS−/− mice. Putative reasons for the rescue by the ET-1 overexpression might include a control of the remodeling of small intra-cardiac arteries by ET-1, a beneficial modification of energy metabolism, as well as a better control of the oxidative stress status (Vignon-Zellweger et al., 2011). Interestingly the level of ET-1 mRNA expression was similar to WT not only in eNOS−/− females but also in ET+/+eNOS−/− mice, despite the ET-1 overexpression. This might indicate that in case of low NO production the ET-1 gene is down-regulated in females.

ET-1 is a potent profibrotic hormone but its effects are limited by NO (Dussaule et al., 2000). In this study, we showed that additional lack of eNOS in ET+/+ mice promotes cardiac interstitial fibrosis independently from sex. In ET+/+ mice, compensatory effects from the NO system have been observed explaining in part the modest cardiac phenotype and in particular the lack of hypertension (Hocher et al., 2004). The fact that combination of ET-1 overexpression and eNOS inactivation results in enhanced interstitial and perivascular fibrosis in ET+/+eNOS−/− mice confirmed these observations.

Collagen I and III belong to the fibrillar type of ECM proteins and are thus responsible for myocardial stiffening and development of systolic and diastolic dysfunctions (Zile and Brutsaert, 2002; Lopez et al., 2006; Diez et al., 2002). Collagen III was not more abundant in both male and female ET+/+eNOS−/− compared to WT, which is in line with the preserved diastolic function in these groups. Non-fibrillar matrix proteins must be responsible for the elevation of Sirius red staining in ET+/+eNOS−/− mice. The other constituents of the matrix are proteoglycans and basement membrane proteins (e.g. laminin, fibronectin). Elevation in laminin expression, but not collagen I, has been reported in cardiac interstitium of ET+/+ 12 month old male mice (Schwarz et al., 2002).

In female eNOS−/− and ET+/+eNOS−/− mice, MMP9 expression was reduced. The sexual hormone 17β-estradiol activates eNOS induced MMP9 expression (Iwakura et al., 2006). The greater amount of estradiol production by females could thus explain the greater effect of eNOS

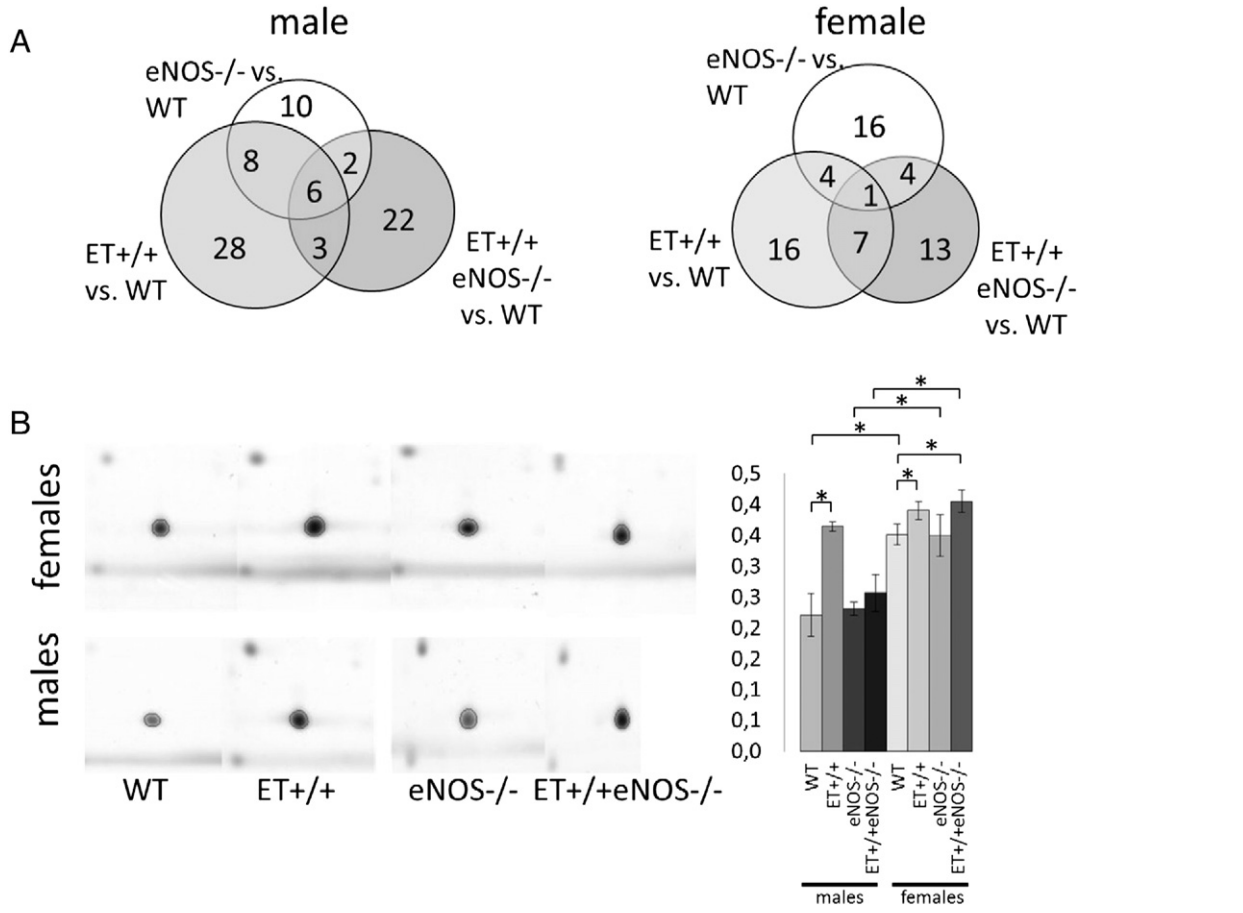

Fig. 6. A: Venn diagrams showing the number of differentially abundant protein species in ET+/+, eNOS−/− and ET+/+eNOS−/− male (left) and female (right) mice compared to WT (n = 6–9). Size of the circle is proportional to the number of protein species. B: 2-Dimensional electrophoresis proteomics pictures of the spot in the hearts of 3 month old males and females. For each group, a portion of a typical silver stained gel from one heart is shown. The spots correspond to a protein species with a specific isoelectric point and molecular weight. Analysis of the digested protein spot by Liquid Chromatography/Electro-Spray Ionization/Mass Spectrometry indicated that this spot corresponds to superoxide dismutase [Cu–Zn]. The diagram shows the relative intensity of the spot. n = 6–9; mean ± sem. Student's T-test *p < 0.05.

deficiency on MMP9 expression and fibrosis development. Moreover, in case of myocardial infarction, MMP9 is less expressed in female than in male mice (Wang et al., 2007). In smooth muscle cells, the activity of MMP9 can be however reduced by eNOS (Gurjar et al., 1999). These conflicting results underline the nonlinear relationship between MMP expression and activity. Indeed, MMP2 activity but not expression is reduced after L-NAME treatment induced NOS inhibition in rat cardiac tissue (Spanikova et al., 2008). Even though the gene expression of MMP2 is regulated by estrogens in cardiac fibroblasts (Mahmoodzadeh et al., 2010), MMP2 activity if not its expression level might differ between male and female eNOS−/− and ET+/+eNOS−/− mice.

Perivascular fibrosis affected female ET+/+ and ET+/+eNOS−/− mice, but not males. Immunostaining techniques used here were unsuitable for quantification of collagen in the perivascular area. Nevertheless, perivascular fibrosis is closely related to inflammation induced perivascular macrophage infiltration (Kai et al., 2005), which is known to be regulated by sex hormones (Georgiadou and Sbarouni, 2009).

Finally, fibrosis is a highly dynamic process. In the left cardiac ventricle, perivascular fibrosis can lead to interstitial fibrosis after extension into the interstitium (Silver et al., 1990). Therefore, fibrillar collagen deposition in the interstitium represents an advanced stage of fibrosis. Taking into account that fibrosis is the consequence of an imbalance between collagen production and degradation by metalloproteases, the elevated collagen I expression and reduced MMP9 expression observed only in females together with development of perivascular fibrosis, suggested that ET+/+eNOS−/− females developed fibrosis more rapidly than males. Sexual dimorphisms in cardiac function can be partially explained by the role of estrogen on collagen expression pattern (Regitz-Zagrosek et al., 2007). Therefore, the differential pattern of ECM between male and female in ET+/+eNOS−/− mice could affect cardiac functions in both sexes differently.

Conclusion

The genetic deletion of eNOS induced diastolic dysfunction, which was stronger in males than in females and a cardiac hypertrophy in males only. The mechanisms by which females were protected from lack of eNOS might include a reduced ET-1 expression and a differential expression pattern of proteins involved in oxidative stress and energy metabolism.

HD version of this video available for your convenience at http://endothelins.com/Educational_Media/ET-13_Lectures/.

Source of funding

This work was supported by grants from the Deutsche Forschungsgemeinschaft and by the European Commission via the Marie Curie Host Fellowship for Early Stage Training (MEST-CT-2005-020268) to Berthold Hocher and Franz Theuring.

Conflict of interest statement

None.

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